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Nondestructive Measurement of Carotenoids in Plant Tissues by Fluorescence Quenching

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ABSTRACT

Carotenoids, compounds valuable for their antioxidant properties in humans and animals, are sometimes present in the bran layers of rice (*Oryza sativa* L.). We developed a nondestructive technique to screen individual rice kernels for the presence of carotenoids in their bran for genetic selection of the carotenoid-containing lines. Most plant tissues are highly autofluorescent. Carotenoids have a high absorptivity for visible light, which allows them to absorb or quench this natural fluorescence. Synchronous fluorescence spectroscopy provides the specificity and sensitivity that allows detection of the changes in fluorescence in a single rice kernel that occur when carotenoids are present. The fluorescence quenching technique identified three rice lines as having carotenoids present in the bran, and three other lines as being carotenoid-deficient. Fluorescence quenching was also used to monitor changes in carotenoid levels in living plants and enabled the measurement of carotenoids within the different colors of a variegated leaf.

Brown rice is rice that has been hulled but not milled so that the bran layers around the endosperm remain. Brown rice is considered to be a healthful food source as it contains a number of nutrients not present or present at low quantities in endosperm (white rice) alone. However, bran also contains a number of antinutritive factors, including phytic acid, trypsin inhibitors, and hemagglutinin (Juliano, 1994). Rice bran also contains carotenoids (Sechi and Rossi-Manaresi, 1958; Saunders, 1990; Juliano and Bechtel, 1994), though not in every cultivar (Juliano and Bechtel, 1994). Concurrently with another study to develop and identify brown rice with reduced levels of antinutritive factors (Rutger et al., 2004), we endeavored to identify brown rice that also contained carotenoids.

The color of rice bran can vary from light tan to very yellow, brown, red, and even purple and nearly black. It is impossible to visually discern a yellowness that may indicate the presence of carotenoids. Thus, the fluorescence spectroscopy technique provides a means for identifying brans containing carotenoids. Carotenoids, which include carotenes and xanthophylls, have a high molar absorptivity and, thus, absorb light efficiently. Conversely, plant tissues are highly autofluorescent; plant cells, when excited by light of the appropriate wavelength, are able to fluoresce without the addition of dyes or other chemicals. Autofluorescence results from lignin, suberin, and other compounds found in healthy cell walls (Cochrane et al., 2000), as well as from a number of compounds that increase in concentration during host–pathogen interactions (Pierce and Essenberg, 1987; Belefant-Miller et al., 1994).

Fluorescence spectroscopy is a highly sensitive technique, used particularly in chemical and physical studies of fluorescent compounds that are often present in organic pollutants. In fluorescence spectroscopy, the sample is illuminated by ultraviolet or visible light (excitation light) and the fluorescent light then emitted by the sample is measured. Synchronous fluorescence (or luminescence) provides an even higher degree of selectivity since both the excitation and emission wavelengths are scanned simultaneously and a signal is generated only where the resulting emission and excitation spectra overlap (Vo-Dinh, 1982). By combining the separate characteristics of autofluorescent emission of visible light by plant tissues with the light-absorbing ability of carotenoids, we developed a rapid, nondestructive technique able to determine the presence of carotenoids in a single rice kernel. This technique also has potential uses for studies of living, rare, or difficult to separate plant tissues.

MATERIALS AND METHODS

Synchronous Fluorescence Spectroscopy

Synchronous fluorescence scans ($\Delta\lambda$ = 10 nm) were performed on a Spex/JY Horiba Fluoromax 3 fluorometer (Jobin Yvon, Inc., Edison, NJ) using slits at 2-nm (excitation) and 2-nm (emission) bandwidths. The solid sample holder with sample was adjusted to produce a maximum signal at λ_{ex} = 400 nm and λ_{em} = 410 nm. After manual optimization of position, the scans were made from 400/410 to 650/660 nm, unless noted otherwise. We were able to screen and analyze 20 to 30 samples per hour.

Fluorescence Quenching by β-Carotene

To demonstrate the mechanism of fluorescence quenching, synchronous fluorescence measurements ($\Delta\lambda$ = 10) were made from 275/285 to 650/660 nm of a kernel of white (milled) rice spotted with 2 μL of chloroform and a kernel of white rice spotted with 2 μL of 10^{-3} M β-carotene dissolved in chloroform. The scan of the rice spotted with β-carotene was mathematically subtracted from the scan of the white, chloroform-spotted rice. The resulting curve shows the difference in emitted light between the two spectra as a result of light absorption by β-carotene.

Integrated Fluorescence Measurements

To ensure that we were detecting changes in light absorbance in our sample measurements, and not just an altered background, we measured the light emitted over a wavelength range

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Abbreviations: NIFI, net integrated fluorescence intensity.

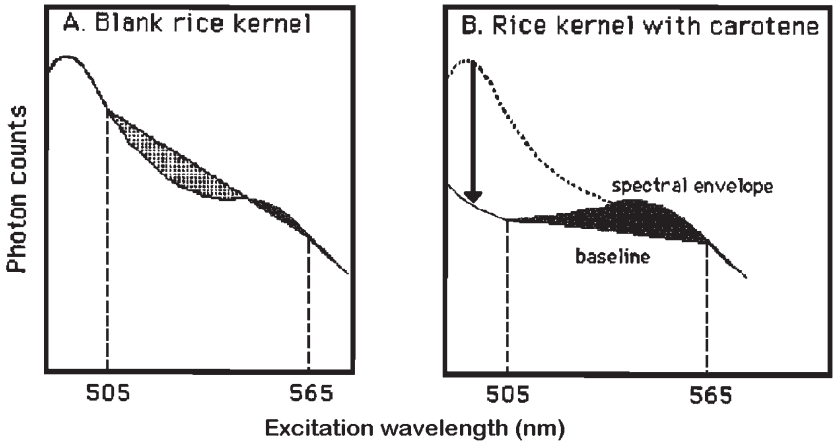

Fig. 1. Illustration of the mathematical integrations of the fluorescence spectra for milled rice (having no carotene) and milled rice spotted with β-carotene. The addition of β-carotene (B) causes a shift downward (arrow) of the spectral envelope at the lower wavelengths since carotenoids, in general, absorb light of wavelengths less than about 525 nm. In the presence of carotenoids, the area above the baseline curve (dark shading in A and B) becomes greater. Changes in the curves of the spectral envelope are measured in comparison to the straight line of the corresponding baseline spectrum by subtracting the area under the baseline from the area under the spectral evelope, thus producing the net integrated fluorescence intensity.

where carotenoid absorption declines rapidly, rather than using only a measurement at a single wavelength. Points on the envelope of the spectrum at 505 nm and 565 nm were connected with a line (Fig. 1). The areas above and below the line were integrated separately; the area above the line was assigned a positive value and the area below the line a negative value. The sum of these values was the net integrated fluorescence intensity (NIFI in Fig. 1). In this wavelength range, the amount of carotene strongly influences the shape of the spectrum since carotene quenches (absorbs) the fluorescent light at the 505-nm end of the analyzed region but not at 565 nm.

Sample Preparation

For the concentration curves of fluorescence quenching by carotenoids, solutions of various carotenoids were spotted onto milled, untreated white rice kernels. The carotenoids β-carotene, lycopene, and lutein were dissolved into chloroform. Their absorptions were measured at their maxima of 460, 487, and 445 nm, respectively, and their concentrations were calculated using the absorption coefficients for that carotenoid (Britton, 1995). Further dilutions were made using absolute ethanol instead of chloroform to minimize residue formation on the rice kernels. Five replicate kernels were made for each β-carotene concentration. One microliter of each β-carotene concentration (10–100 ng μL^{-1}) was spotted onto a rice kernel and allowed to dry for a minimum of five minutes in the dark before scanning with the fluorometer. The average value obtained for the blank (unspotted, milled rice) was added to the values obtained.

Rice kernels for bran measurements were provided by researchers at Dale Bumpers National Rice Research Center and the University of Arkansas Rice Research and Extension Center, both in Stuttgart, AR. Preliminary experiments indicated that most brown rice samples were a mixture of individual kernels that were either highly positive or negative for carotenoids. Therefore, we identified cultivars with bran that were exclusively carotene-containing or carotene-deficient. The lines selected were Cocodrie, Drew, and Wells (U.S. cultivated lines) as being uniformly carotene-deficient and Stg-S and LA-3 (weedy red rice accessions) and Liao 8801 (Chinese germplasm) as carotene-containing. Fluorescence quenching measurements were made of 10 kernels from each line.

Mature variegated leaves of the ornamental bush *Eunonymus japonica* 'Auroo-Marginata' were picked fresh for fluorescence quenching measurements. Measurements were made from visually similar areas of yellow or of light, medium, or dark green in each of five leaves. A bicolor ear of fresh sweet corn (*Zea mays* L.), purchased locally, provided both the yellow and white kernels for fluorescence quenching measurements. The corn pericarp and/or outer endosperm of 10 replicate kernels were removed manually for measurement of the different tissues.

Etiolated rice plants were obtained by sprouting and maintaining rice plants in a cabinet in a dark room. Other seeds were sprouted and maintained in the greenhouse at the same time. The fully emerged second leaf was tested while still attached to the plant. The etiolated plants in their containers were small enough to fit into the sample area of the fluorometer. The leaf to be tested was positioned on the solid sample holder with adhesive tape outside the optical path. The etiolated plants were put in a greenhouse after the initial reading and measurements were made at 1-h intervals thereafter. One carotenoid measure was made from each of five replicate plants at each time point.

Rice kernels, which are smaller than the chamber of the solid sample holder, were fixed into place using black modeling clay that tested negative for fluorescence. Larger plant samples, such as the variegated leaf of *E. japonica* and the corn kernel, were cut to size to fit the solid sample holder. The results from the replicates at each test point were averaged and the standard deviation from the mean computed.

Extraction and Absorption Measurements of Carotenoids from Bran

Bran was collected by milling the brown rice on a Grain Testing Mill (Satake, Tokyo) for 80 s. Each 0.3-g sample of bran was ground using a mortar and pestle in 0.05% butylated hydroxytoluene (BHT) in acetone. The acetone/bran extraction was sonicated for 30 s and then allowed to sit at room temperature in the dark for 15 min. The extraction was centrifuged and the carotenoids in the solvent were concentrated under vacuum at 30°C. Absorption measurements were made of the extract resuspended in hexane on a Jenway 6505 UV/Vis spectrophotometer (Jenway, Essex, UK). Absorbances at 450 nm were used to calculate carotenoid concentration using

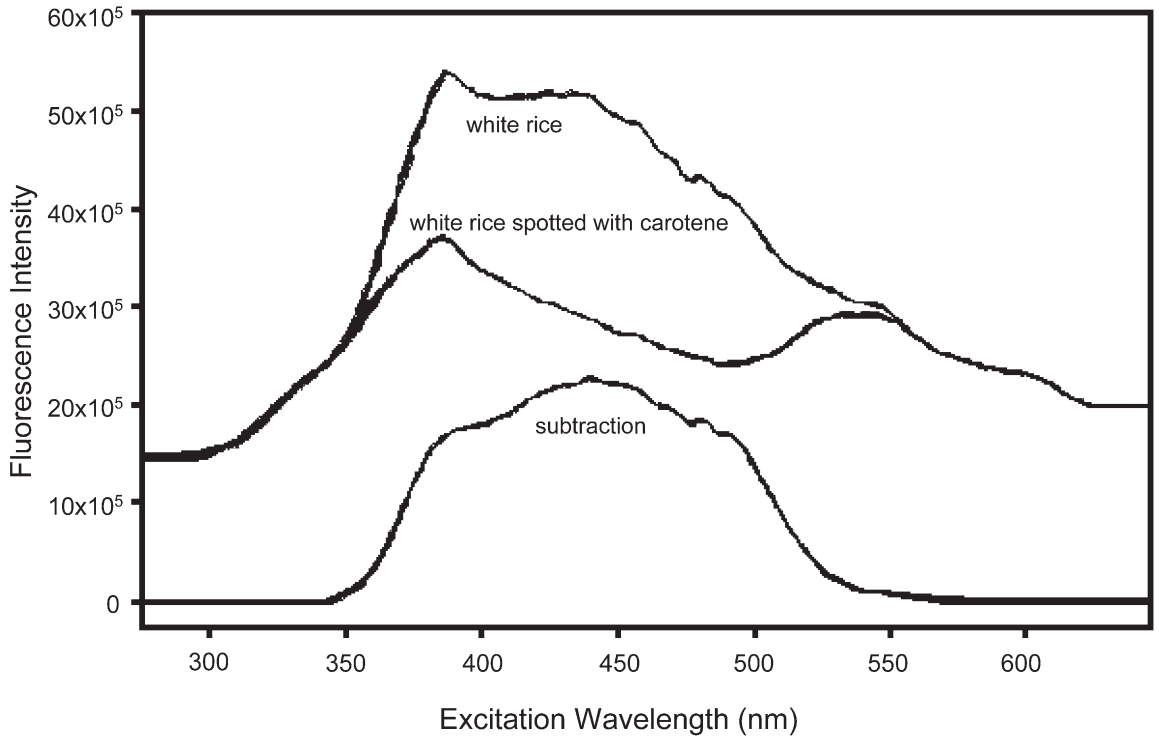

Fig. 2. Demonstration of the changes in autofluorescence that occur during fluorescence quenching. The natural autofluorescence of milled white rice is the *white rice* line. The scan of *white rice spotted with carotene* was subtracted from the scan of *white rice*. The resulting *subtraction* line is the absorption spectrum of the β-carotene in the dry endosperm matrix. The net integrated fluorescence intensity is obtained from the 505- to 565-nm region of a fluorescence spectrum.

an absorption coefficient of 2500 (1%) (1 cm^{-1}) (Schiedt and Liaaen-Jensen, 1995).

RESULTS AND DISCUSSION

Carotenoids are known to be absent in white rice (Hoa et al., 2003), and so white rice was used as a blank substrate for establishing the parameters for carotenoid detection. Figure 2 shows the native fluorescence of an intact rice endosperm (white rice) along with the absorption, or quenching, of this fluorescence by β-carotene applied to the rice (spotted). Subtraction of the quenched from the unquenched synchronous fluorescence spectrum reveals the shape of a typical absorption spectrum of carotenoids (subtraction), indicating that the quenching mechanism is absorption of light by the carotenoids.

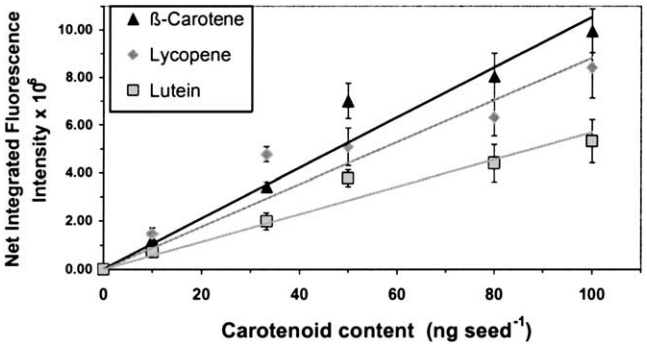

Fig. 3. Dose response curves of net integrated fluorescence intensities from fluorescence quenching measurements of known carotenoids. Measurements were made of white rice kernels spotted with 1 μL of five different concentrations of three carotenoids. Bars indicate standard deviations.

The NIFI, a relative measure of carotenoid amount, increased linearly with increasing amounts of the carotenoids, β-carotene, lycopene, and lutein (Fig. 3). The fluorescence quenching technique was optimized for β-carotene so while the technique is most responsive to changes in its level, the other carotenoids are detected as well.

The bran of the U.S. rice lines tested by fluorescence spectroscopy had no detectable carotenoids, while the Chinese germplasm and the red rice bran contained carotenoids (Table 1). These values for the different rice lines were in good relative agreement with the values obtained by extraction (Table 1).

Although our measurements of carotenoids in rice bran are only intended as relative measures, some level

Table 1. Comparison of a nondestructive technique (net integrated fluorescence intensity, NIFI) with solvent extraction for the measurement of carotenoid levels in rice bran.

	Measurement technique	
Rice line	Fluorescence quenching	Extraction
	NIFI ± SD × 10⁶†	Carotenoids, μg g^{-1}
Stg-S	2.4 ± 0.7	2.6
LA-3	1.6 ± 0.4	1.8
Liao 8801	1.3 ± 0.6	1.5
Wells	0.1 ± 0.2	n.d.‡
Cocodrie	0.1 ± 0.2	n.d.
Drew	−0.1 ± 0.2	n.d.

† Multiply the reported numbers by this to obtain the actual numbers.
‡ n.d., not detectable.

Table 2. NIFI values for various intact plant tissues having varying carotenoid levels using quenching of autofluorescence.

Plant tissue	NIFI ± SD ($\times 10^6$)†
Corn kernel	
White, endosperm through pericarp	8.4 ± 5.4
White, outer endosperm	6.7 ± 5.2
White, inner endosperm	2.8 ± 1.6
Yellow, endosperm through pericarp	21.4 ± 9.6
Yellow, outer endosperm	20.8 ± 12.6
Yellow, inner endosperm	10.4 ± 3.4
***Eunonymus japonica* variegated leaf**	
Light green	6.9 ± 6.4
Medium green	9.5 ± 7.9
Dark green	10.3 ± 9.2
Yellow	16.0 ± 7.3
Rice leaf	
Light-grown	25.0 ± 3.9
Dark-grown (etiolated)	19.4 ± 4.7
Etiolated and 1 h of light	21.6 ± 4.3
Etiolated and 2 h of light	30.2 ± 5.5
Etiolated and 3 h of light	26.6 ± 9.3

† Multiply the reported numbers by this to obtain the actual numbers.

of quantification is possible since a background tissue is available in the form of carotene-less rice endosperm. To provide an estimation of the actual content of carotenoids in bran from the unitless values obtained by fluorescence quenching, values from Fig. 3 were used as a standard curve to estimate the carotenoid levels for the carotene-containing cultivars. These cultivars were measured as having 1.3 to 2.4 $\times 10^6$ NIFI (Table 1), yielding a very approximate range of 11 to 23 ng carotenoids on a single kernel (Fig. 3). This would be about 0.55 to 1.15 μg carotenoids g^{-1} brown rice (using 20 g brown rice per 1000 kernels, but not extrapolating the area read by the fluorometer to the whole surface area) or 6.1 to 12.7 μg carotene g^{-1} bran, assuming 9% of brown rice to be bran (Saunders, 1986). This range is near the values observed previously of approximately 4 μg g^{-1} bran (Sechi and Rossi-Manaresi, 1958; Saunders, 1990; Juliano and Bechtel, 1994) and the value obtained here by extraction of approximately 2 μg g^{-1} (Table 1).

Measurement by fluorescence quenching of the relative carotenoid levels present in plant tissues verified observations that have previously been made. Most of the color of yellow corn is in the outer portion of the endosperm (Blessin et al., 1963), which can be measured using fluorescence quenching through the transparent pericarp (Table 2). As expected, the inner endosperm of the yellow corn had a lower carotenoid level than the outer endosperm, and carotenoid levels overall were reduced greatly in white corn.

The fluorescence quenching technique provides an ideal method to detect the presence of carotenoids in difficult to isolate tissue portions such as the rice bran layer, which is only a few cells thick. Other plant materials also have colored parts that are difficult to dissect or separate. For example, using fluorescence quenching, the yellow to green areas of a variegated leaf of *E. japonica* were measured but without the need to separate each colored section of the leaf (Table 2).

Our measurement technique can also be used to monitor carotenoid levels in living plants. We measured carotenoid levels in attached etiolated rice leaves over several hours of exposure to light (Table 2) and, as has been previously observed (Barry et al., 1991), the carotenoid level in the light-grown leaves was similar to those of the etiolated leaves during the 3 h of light exposure, although the leaves varied in appearance from pale yellow-green to dark green.

The fluorescence quenching technique described here obviates the need for extraction and chromatography to obtain relative amounts of carotenoids in a plant tissue sample. This nondestructive and sensitive procedure uses the autofluorescence inherent in plant tissue to enable measurement of carotenoids in rare samples, difficult to isolate tissue portions, and in living tissues. Fluorescence quenching is an effective and rapid screening technique for carotenoids in rice bran. An obvious tradeoff for a nondestructive technique is the impossibility of identification of specific pigments. For screening large amounts of material or for conditions where changes in the specific carotenoid makeup is not of interest, the speed and nondestructive nature of this assay method is valuable.

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